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Fiber-Optic DNA Sensor for Fluorometric Nucleic Acid Determination

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Single-stranded deoxyribonucleic acid (ssDNA) thymidylic acid icosanucleotides (dT_{20}) were synthesized on the surfaces of derivatized quartz optical fibers to create an optical DNA biosensor. The synthesis made use of an automated solid-phase synthesizer and phosphoramidite synthons. The covalently immobilized oligomers were found to hybridize with complementary ssDNA (cDNA) or ssRNA (cRNA) from solution, and the device was regenerable for multiple cycles of application. Hybridization on optical fibers was detected by the use of the fluorescent DNA stain ethidium bromide (EB). The procedure used hybridization assay techniques and provided a detection limit of 86 ng·mL^{-1} cDNA and a sensitivity of 200% fluorescence intensity increase per 100 ng·mL^{-1} of cDNA, with one cycle of hybridization analysis requiring 45 min. The sensor has been observed to be regenerable (minimum of five cycles) and to sustain full activity after prolonged storage times (1 year), harsh washing conditions (sonication), and sterilization (autoclaving). The extent of hybridization between the immobilized and complementary nucleic acid strands was determined by UV absorbance thermal denaturation studies wherein all 20 bases on each strand of the nucleic acid were found to be involved in duplex formation.

The goal of our work is to investigate rapid nucleic acid detection methods for development of biosensors which could potentially provide results in minutes, and in conjunction with amplification techniques (such as PCR) that could be used to achieve high sensitivity. A number of methods of cell identification which are relatively rapid and reliable have been developed. These newer methodologies are commonly based on two approaches: investigations of extracellular properties and examinations of intracellular properties. Immunoassays, metabolic light addressable potentiometric sensors (LAPS), and acoustic resonance sensors which investigate sample microviscosity provide examples of methods that have been used to detect and identify microorganisms on the basis of their extracellular properties.[1–3] Perhaps the most common method for selective detection of target organisms by extracellular techniques is to use antibodies which selectively recognize and bind to a specific region of the cellular membrane of the target.[4,5] The major limitation of sensors that use protein-based biorecognition elements lies in the fact that over short time periods (days to weeks), the tertiary structure of most proteins removed from their natural environments becomes denatured, causing loss of the selective binding activity.[6] A commonly investigated intracellular property is the nucleic acid sequence present within cells.[7–9] The analysis of nucleic acids has proven to have advantages over plating/culture or other methods which investigate extracellular properties. In many cases, isolation of microorganisms is not required, both live cells or the contents of dead cells may be detected, and excellent selectivity can be obtained, to the point where genetic mutations may be investigated. As such, nucleic acid analysis has now become commonplace for clinical laboratory testing.[10–14]

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The key to nucleic acid detection strategies lies in the use of nucleic acid probes, which are normally composed of DNA due to its greater resistance to hydrolysis. Nucleic acid probes are typically short sequences (10–30-mers) of single-stranded nucleotides that are capable of hybridizing with specific regions of a target nucleotide sequence unique to a particular species or genus of organism.[15] The oldest and most routinely used types of hybridization assays for nucleic acid analysis are solid-phase or filter hybridizations. An excellent review of hybridization assay techniques is given by Wolcott.[15]

With the recent advent of DNA probe technology, a number of selective oligomers which interact with the DNA of important biological species have been identified.[16–19] These probes are a form of biorecognition element which are highly selective and stable and can be easily synthesized in the laboratory,[20–22] in contrast to other commonly used biorecognition elements, such as enzymes and antibodies.[6,23–25] DNA biosensors that are presently being developed are largely based on piezoelectric,[26–29] electrochemical,[30–32] and, more recently, optical transducers.[33–35]

The work herein reports studies directed toward the development of biosensors for direct analysis of DNA hybridization by use of an optical fiber and a fluorescent intercalating agent. The method involved covalent immobilization of ssDNA onto optical fibers by first activating the surface of the quartz optical fibers with a long-chain aliphatic spacer arm, followed by automated solid-phase oligonucleotide synthesis. Detection of double-stranded DNA (dsDNA) at the fiber surface after hybridization between immobilized ssDNA and complement ssDNA (cDNA) was achieved by staining the complex with a fluorescent intercalating dye. It is shown that the fluorescence response of the intercalated fluorophore could be monitored by using optical fibers in a total internal reflection configuration, with the fluorescence intensity being directly proportional to the amount of complement DNA present in solution. Further investigations of the optical sensor involved determination of response to cRNA, response time, staining time, the effect of altering fluorophore concentration, and sensor regeneration methods. The completeness of duplex formation was also investigated, wherein the thermal denaturation profile of immobilized dsDNA was compared to that of dsDNA in solution.

EXPERIMENTAL SECTION

Chemicals. Fast oligonucleotide deprotection riboadenosine phosphoramidite synthon, FOD-rA, used for the assembly of rA_{20} (or cRNA), was purchased from Applied Biosystems Inc. (ABI, Mississauga, Canada). All other chemicals used for the assembly and the fluorescence investigations of the prototype fiber-optic DNA biosensor have been previously reported.[33]

Apparatus. All DNA and RNA synthesis was done according to the well-established β-cyanoethylphosphoramidite method with an Applied Biosystems 381A DNA synthesizer on various solid substrates. These substrates were of three types: controlled-pore glass beads, quartz optical fibers, or planar quartz wafers. All fluorometric investigations of nucleic acid hybridization were done using oligonucleotides grown on quartz optical fiber substrates, and DNA synthesized on quartz wafers was used for thermal denaturation studies of immobilized duplexes. Controlled-pore glass, functionalized with long-chain alkyl amine (LCAA) spacers terminated with an adenosine nucleoside, was used for the synthesis of all analyte sequences. Dimethoxytrityl cation released from each deprotection step of oligonucleotide synthesis was quantitatively measured by absorption spectroscopy at 504 nm using a Hewlett Packard 8452A diode array spectrometer (Hewlett Packard Corp., Palo Alto, CA) to determine both the amount of coverage of dT_{20} on the optical fibers and quartz wafers and the coupling efficiency of all nucleic acid syntheses. Measurement of absorbance at 260 nm was used to quantify the amount of purified oligomers and to investigate the melting characteristics of immobilized duplexes in conjunction with a thermostated cuvette housing. Thermal denaturation studies of aqueous phase oligomers were conducted using a Varian Cary I UV–visible spectrophotometer (Mulgrave, Australia) equipped with Peltier temperature controller. Data were collected on a PC with software supplied by the manufacturer (Cary 1.3e).

The instrument used for fluorescence intensity measurements was based on an inverted fluorescence microscope, as designed by Krull and co-workers,[36] and is shown in Figure 2. The output from a Coherent Innova 70 CW argon ion laser (Coherent Laser Products, Palo Alto, CA) operated at 488 nm and 10 mW power was directed into a Zeiss IM inverted microscope (Carl Zeiss, Oberkochen, Germany), which was mounted such that the objective lens was aimed downward. Fluorescence emission from doped DNA membranes would pass through the dichroic mirror (495-nm cut-off) to a low light level Dage-MTI SIT 66 video camera (Dage-MTI Ltd., Michigan City, IN), from which a video image could be obtained. The camera was linked to an Oculus 300 video grabber card (Coreco Inc., Cleveland, OH), which digitized the image (480 × 512 pixels) for storage and processing on a PC. A Zeiss Model 477414 photomultiplier tube (PMT) was attached to the microscope for collection of fluorescence intensity from immobilized membranes. Fluorescence could be directed either to the SIT camera or to the PMT using a rotatable mirror. The

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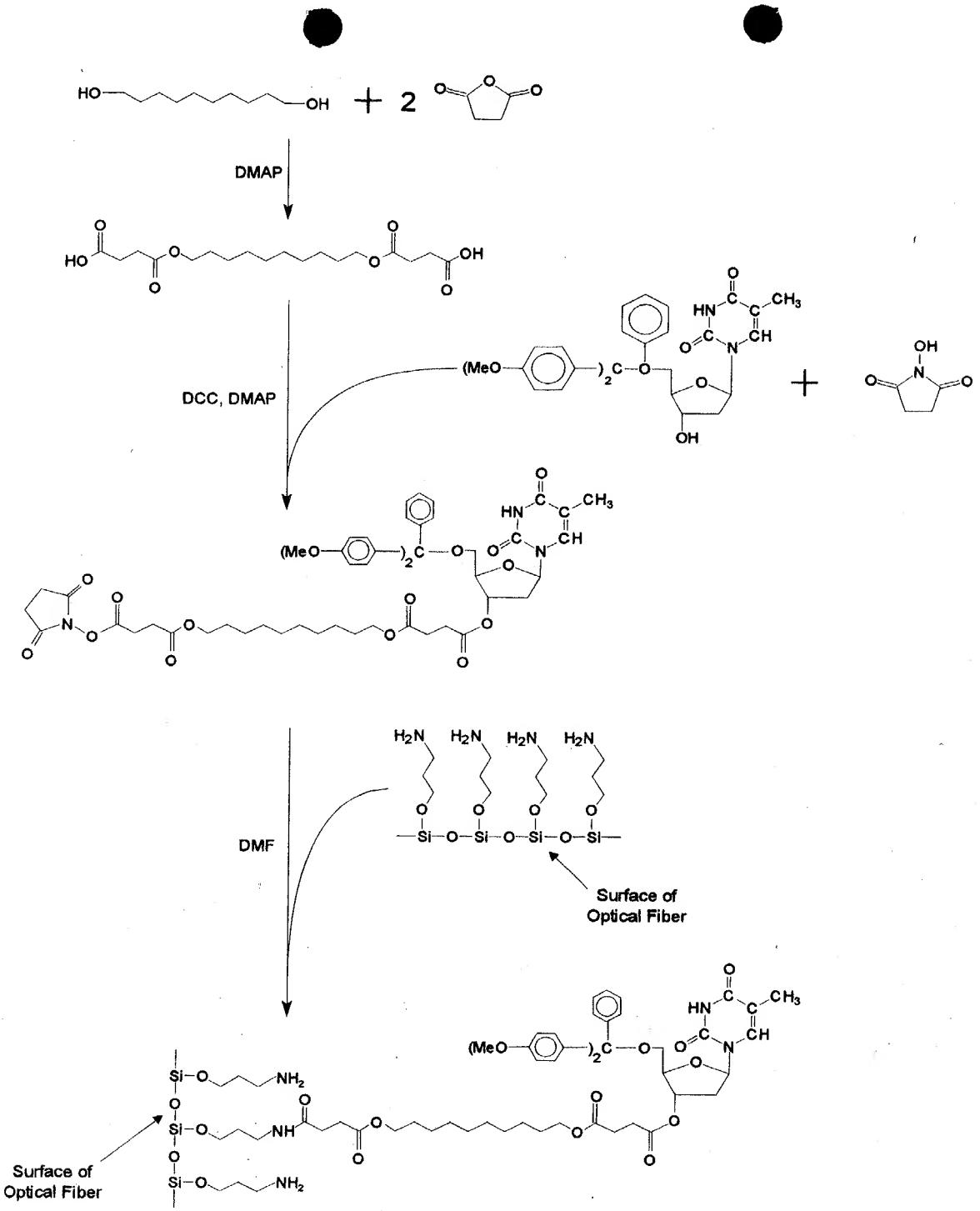

Figure 1. Synthetic scheme used to activate the surface of the quartz optical fibers and planar quartz wafers with long-chain aliphatic spacer molecules terminated with a 5′-*O*-dimethoxytrityl-2′-deoxythymine nucleoside.

microscope, PMT, and camera were mounted on a gas-damped vibration isolation table (Melles Griot, Rochester, NY).

Synthesis of DNA Membranes. A suitably functionalized substrate surface was required in order to permit the coupling of nucleotide monomers before automated solid phase synthesis of oligonucleotides could be done. The substrates used for the work herein were long-chain alkylamine derivatized controlled-pore glass beads (LCAA-CPG), quartz optical fibers, and planar quartz wafers. Prior to introduction onto the automated synthesizer, the LCAA-CPG beads were functionalized with 5′-*O*-dimethoxytrityl nucleosides using the method of Damha and co-workers.[37] Functionalization of quartz substrates for automated solid-phase DNA synthesis was based on the method of Arnold et al.,[38] as has been described previously,[33] and is shown schematically in Figure 1.

Solid-Phase Phosphoramidite Synthesis of Oligonucleotides. Sequences of DNA and RNA were grown on CPG beads, optical fibers, and planar quartz wafers using the β-cyanoeth-

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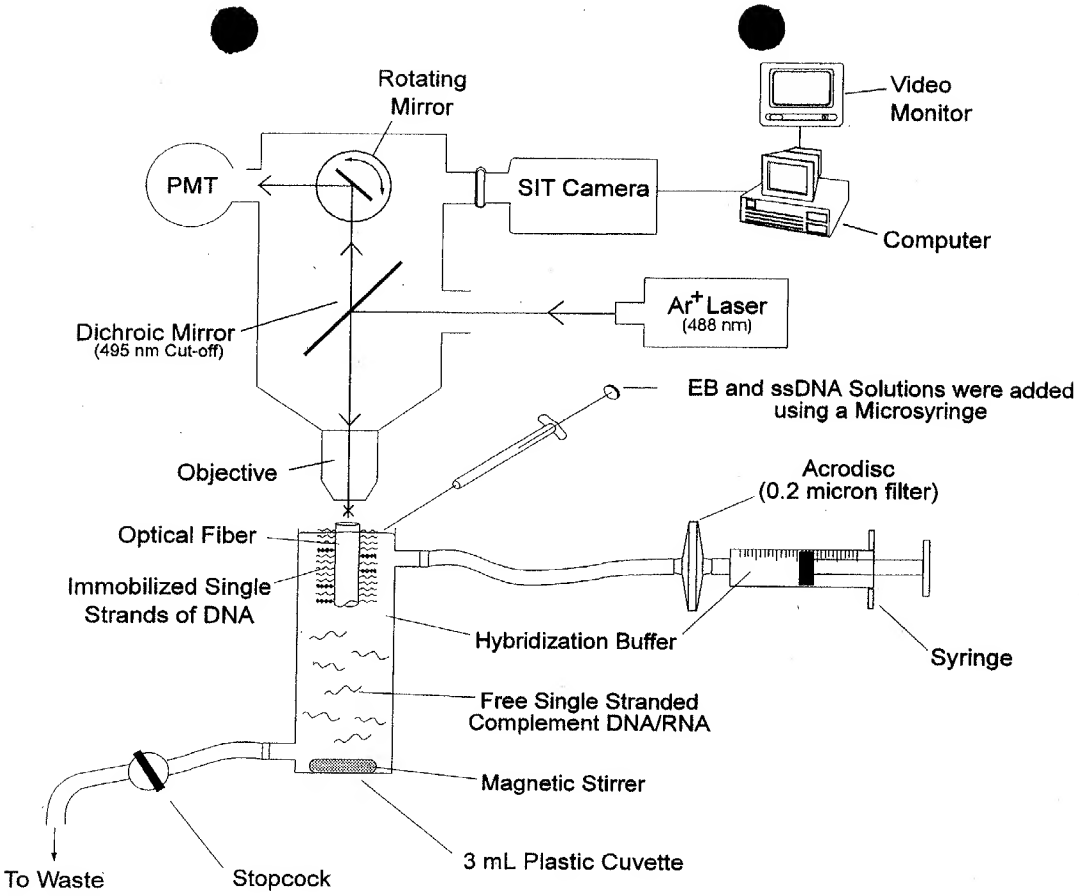

Figure 2. Instrumentation used to measure fluorescence intensity from optical fibers coated with immobilized DNA.

ylphosphoramidite method and an automated DNA synthesizer. All phosphoramidite synthons were stored at −20 °C and were desiccated under vacuum and over P_2O_5 before use. Immediately prior to introduction into the automated synthesizer, phosphoramidite reagents were dissolved in anhydrous acetonitrile to provide a 0.1 M solution of DNA or a 0.15 M solution of RNA phosphoramidite synthons. All other reagents for solid-phase synthesis of oligonucleotides were prepared as reported by Damha et al.[39,40] Synthesis of oligomers onto the optical fibers was carried out at the 0.2-μmol scale with a pulsed-delivery cycle in the "trityl-off" mode (ABI) using extended nucleoside coupling times (2 min instead of 30 s). For introduction onto the DNA synthesizer, surface-derivatized optical fibers ~15 mm long were placed into an emptied oligonucleotide purification cartridge (OPC, 15 mm × 6 mm i.d.; ABI), with the dead volume taken up by finely divided pieces of polypropylene. The end filter papers were replaced, and the column ends were crimped closed using aluminum seals. Deprotection of the phosphate blocking groups from the immobilized oligomers was achieved by standing the fibers in a solution of 2:3 triethylamine/acetonitrile at room temperature for 1.5 h.[41]

The nonadecamer of random base composition (dR_{19}) was prepared using the same synthetic routine as for the dA_{20}, with the exception that all four phosphoramidite reagents were simultaneously introduced into the column at each coupling step. Standard deprotection with aqueous ammonia (29%, 1.5 mL, 48 h) was used to liberate the DNA oligomers from the CPG support and to remove the base protecting groups. In the case of rA_{20}, deprotection of the phosphate and heterocyclic blocking groups and cleavage from the CPG support was done by treating the oligomers with ethanol/aqueous ammonia (4:1, 1.5 mL, 48 h, 25 °C).[40] The aqueous solution containing the oligonucleotides was then collected and evaporated to dryness, and the residue was treated with 300 μL of an anhydrous solution of 1 M tetra-*n*-butylammonium fluoride in THF overnight at room temperature. After the incubation time, the reaction was quenched by adding 1 mL of water to the reaction mixture.

Purification of Oligonucleotides. Following deprotection, the solution containing the oligomers was desalted by size exclusion chromatography using Sephadex G25F. The fractions containing oligomers (as determined by absorption spectroscopy, A_{260}) were evaporated to dryness. The oligomers were then purified by polyacrylamide gel electrophoresis. After electrophoretic separation, the portions of the gel containing full-length oligomers were isolated, crushed, placed in 1.5 mL of sterile water, and incubated for 16 h at 37 °C in order to extract the oligonucleotides from the gel. The solution was again desalted by size exclusion chromatography, and the concentration of oligomer in each fraction was determined by A_{260}.

Procedures for Biosensor Characterization. *Trityl Cation Assay.* All syntheses were evaluated by spectroscopic quantitation of trityl cation released during the trichloroacetic acid (TCA) treatment steps of the automated synthesis. The collected

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fractions of trityl cation were diluted with 2.0 mL of 5% TCA in 1,2-dichloroethane immediately before absorbance measurements were made. Absorption at 504 nm was measured in order to determine the concentration and the total number of trityl cations released during each deprotection step of the synthesis. In this way, the total number of oligomers successfully grown on the solid supports was determined.

Fluorescence Investigations of the Optical Sensor. For each experiment, a fiber coated with ssDNA was selected at random from the batch of fibers (~25) on which ssDNA was grown and was positioned under the objective of the fluorescence microscope. In this orientation, incident laser radiation entered the fiber at the proximal terminus and was totally internally reflected. The majority of the fiber was submerged in a hybridization buffer solution consisting of 1.0 M NaCl and 50 mM sodium phosphate (pH 7.0) in sterile water. Hybridization buffer was passed through an acrodisk filter immediately prior to introduction into the cuvette. The intensity of fluorescence emission from the fiber was measured by directing the emitted light to the PMT.

Thermal Denaturation Investigations of Aqueous dT_{20} with Aqueous dA_{20}. Equimolar amounts of each oligomer in hybridization buffer (1 M NaCl, 10 mM PO_4^{3-}, pH = 7.0) were mixed so that the final concentration was approximately 1 μM in each strand. Prior to thermal melt studies, the oligonucleotide mixture was heated briefly to 80 °C and slowly cooled to 20 °C in order to hybridize all of the strands. The samples were held at the low-temperature limit for 15 min before the melt studies were initiated, to allow for thermal equilibration. The temperature was then ramped at 0.5 °C intervals at a rate of 0.5 °C/min while the absorbance was recorded at 260 nm.

Melt Curve Investigations of Immobilized dT_{20} with cDNA. dT_{20} immobilized on a planar quartz wafer was hybridized with complementary dA_{20} sequences by immersing the wafer in a 56.8 $\mu g \cdot mL^{-1}$ solution of dA_{20} at 85 °C and allowing the immersed wafer to cool to room temperature (25 °C). The wafer was then removed from the cDNA solution and washed with room temperature hybridization buffer solution. The wafer was then suspended in a quartz cuvette that was placed in the temperature-controlled cuvette housing of the UV–visible spectrometer. The wafer was adjusted so that it rested in the path of the light beam. The dead volume beneath the wafer was taken up by inert packing material. Absorption spectra were collected at ~2 °C increments of temperature in the range from 29 to 76 °C. The temperature in the cuvette was set by programming an external circulating bath to a specific temperature, and the temperature of the buffer solution surrounding the quartz wafer was quantitatively measured using an immersible thermistor. Measurements of absorption at each temperature were done by integrating 100 spectra in the wavelength range between 220 and 320 nm.

Fluorescence Investigations of the Optical Sensor. For each experiment, a fiber coated with ssDNA was selected at random from the batch of fibers (~25) on which ssDNA was grown, and this fiber was positioned under the objective of the microscope. In this orientation, the incident laser radiation entered the fiber at the proximal terminus and was totally internally reflected. The majority of the fiber was submerged in a hybridization buffer solution consisting of 1.0 M NaCl and 50 mM sodium phosphate (pH 7.0) in sterile water. Hybridization buffer was passed through an acrodisk filter immediately prior to introduction into the cuvette. The intensity of fluorescence emission from the fiber was measured by directing the emitted light to the PMT.

Hybridization Studies of DNA Membranes. Hybridizations were done by adding various volumes of aqueous solutions containing purified dA_{20} (2.75 $ng \cdot \mu L^{-1}$ or 56.8 $ng \cdot \mu L^{-1}$), dR_{19} (60 $ng \cdot \mu L^{-1}$), or rA_{20} (3.8 $ng \cdot \mu L^{-1}$) to a plastic cuvette containing the suspended fiber in fresh hybridization buffer at 85 °C. The solution was allowed to cool to room temperature (25 °C), after which time the fiber was flushed with 60 mL of hybridization buffer (25 °C). The temperature of the contents of the cuvette was known at all times by use of an immersible thermistor.

Staining of double-stranded nucleic acids was done by injecting various volumes of a 1 $mg \cdot mL^{-1}$ aqueous solution of ethidium bromide (EB) into the cuvette and allowing the solution to stand for 15 min, followed by washing the fiber by flushing the cuvette with 60 mL of fresh hybridization buffer (25 °C).

Regeneration of ssDNA at the surface of the optical fiber and removal of EB was done by flushing 30 mL of the hot (85 °C) buffer through the cuvette over a time of about 30 s. The system was then allowed to stand for 5 min, after which time the cuvette was drained. Hot (85 °C) deionized water (30 mL) was passed over the entire fiber surface while the cuvette was continually drained. The cuvette was then flushed with an additional 30 mL of hot buffer to wash away any remaining cDNA strands.

RESULTS AND DISCUSSION

An automated DNA synthesizer was used to grow thymidylic acid icosanucleotides (dT_{20}) on the surface of activated optical fibers via the β-cyanoethylphosphoramidite method.[42] The use of an automated synthesizer to grow DNA on the optical fiber substrates provides many advantages over conventional techniques of DNA immobilization. Conventionally, nucleic acid strands are adsorbed onto a suitable support (usually nylon or nitrocellulose) with little known about strand orientation. The use of an automated synthesizer provides control of the oligomer sequence and strand orientation when experiments are done with quartz substrates functionalized with spacer molecules. Covalent immobilization of probe sequences via the strand terminus is known to provide hybridization rates 10–100-fold faster than those of oligonucleotides adsorbed onto solid supports (e.g., nitrocellulose) with no control over strand orientation and attachment sites.[43] As such, the rate of hybridization between end-immobilized oligomers with aqueous complement sequences has been observed to approach (0.2–1.0-fold) that of solution-phase hybridization kinetics. It has also been reported by Zhang et al.[44] that the use of a linker (≥28 Å in length) between the support and the immobilized strand provides hybridization efficiencies 4 times greater than that when no linker has been used to immobilize oligonucleotides. This is in accord with the report of Beaucage et al.,[45] wherein it was stated that nucleotide supports of lengths of at least 25 atoms are required to achieve high (≥98%) coupling yields during automated solid-phase oligonucleotide synthesis.

The method of Arnold and co-workers[38] was used for the activation of the quartz wafers and optical fibers, whereby 25-atom-

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long spacer molecules terminated by a dimethoxytrityl-protected nucleoside were immobilized onto the cleaned surfaces. This method was chosen for a number of reasons. The length of the spacer between the substrate and the first nucleoside was suitably long so as to provide high hybridization rates and efficiencies and to make the environment of the terminal nucleoside sufficiently fluid to permit efficient coupling with successive nucleotide monomers during automated phosphoramidite synthesis. The synthetic scheme required inexpensive chemicals, was facile to perform, and was done as a one-pot procedure, wherein product isolation and purification were obviated. Because the linker was terminated by a protected nucleoside, any reactive sites on the support which would lead to the production of unwanted side products during automated synthesis could be eliminated by treating the derivatized supports with acetic anhydride or chlorotrimethylsilane prior to synthesis.[46] Lastly, the coverage of linker on the support was easily determined by the amount of trityl cation released during the first TCA deprotection step of the automated synthesis.

The coupling efficiency of each cycle of the automated synthesis was determined by measuring the amount of trityl cation released during each cycle of synthesis. The results of the trityl cation assay have indicated that, on average, 1.1×10^{14} molecules of ssDNA have been grown on each optical fiber.[33] As no discernible decrease in the amount of trityl cation released during successive deprotection steps was observed, a coupling efficiency of 98% (minimum suggested by ABI) or better (as extended coupling times were used) was achieved.

Strategy for the Preparation of Optical Sensors. Triethylamine was used for the removal of β-cyanoethyl protecting groups on the internucleotidic phosphotriester moieties of oligonucleotides grown on quartz substrates.[41] This procedure is known to cause quantitative loss of the phosphate protecting groups via a β-elimination mechanism[47] while not cleaving the ssDNA from the optical fibers. Ammonia treatment of the immobilized oligonucleotides was avoided by choosing an all-thymine base sequence. Thymine does not contain primary amine functionalities which would require protection during oligonucleotide synthesis.

A detection scheme based on fluorescence was chosen because fluorescence techniques provide high sensitivity and detailed information about structure at the molecular level.[48] The fluorophore chosen to investigate hybridization in DNA monolayers was ethidium bromide. The ethidium cation (3,8-diamino-6-phenyl-5-ethylphenanthridium) is a fluorescent compound which strongly associates with dsDNA by intercalation into the base stacking region and, in some cases, the major groove of the double helical structure.[49–52] The ethidium cation is particularly well suited for investigations of nucleic acid hybridization because the quantum yield of the dye increases as much as 100-fold when the dye is intercalated into the base stacking region.[53] Moreover, the binding affinity and the fluorescence enhancement of the dye are independent of base composition, and intercalation of the ethidium cation is known to increase duplex stability (the two 3,8-amino substituents hydrogen bond with the internucleotide phosphate groups on each of the DNA strands, whereas other intercalators may significantly decrease duplex stability).[54] EB has an absorption maximum of 510 nm, which is sufficiently close to the output wavelength of 488 nm of the Ar^+ laser used in the fluorescence microscope to excite the fluorophore. The dye has an emission maximum of 595 nm when bound to DNA, which is a sufficiently large Stokes shift to prevent inner filter effects and to make separation of the emission radiation from the excitation radiation straightforward by the use of a dichroic mirror.[55]

Although both planar wafers and optical fibers may be used for investigations of membrane-based sensors, optical fibers were chosen for development of sensors. Optical fiber supports are particularly advantageous due to their small size, high light transmission capability, and ability to allow total internal reflection (TIR) of light, which can produce evanescent electric fields. The intrinsic mode arrangement was used to monitor fluorescence emission from the surface of optical fibers.

Typically, the evanescent wave propagates beyond the surface of a fiber, with a penetration depth ranging from 200 to 400 nm for visible light. Because the refractive index of a monolayer of organic media ($n_{\text{monolayer}} \cong 1.46–1.5$)[56] is very similar to that of the substrate ($n_{\text{quartz}} = 1.46$),[57] direct excitation of fluorophores in the monolayer may occur, as the boundary for TIR would be the monolayer–solution interface. Light emitted from fluorophores (after evanescent or direct excitation) at the surface of the fiber is preferentially coupled back into the fiber.[58] Increasing the length of coated fiber results in a greater optical path length and better sensitivity. This method is most suitable for the study of ultrathin films, where the intensity of the evanescent wave is near its maximum, thereby increasing the signal to noise ratio as interferences from the bulk environment are largely avoided. In accord with the characteristics of an ideal biosensor, optical fibers also provide a compact and rugged sensing device and offer the ability to do remote spectroscopic measurements.[58,59]

Hybridization Studies of Covalently Immobilized DNA Membranes. *Hypochromicity and Melt Curve Thermodynamics.* The transition between an ordered duplex state and the disordered denatured state for systems of complementary nucleotides can be monitored and analyzed by UV–visible absorbance spectroscopy to determine the duplex melting temperature (T_m). The extent of hybridization (i.e., the number of base pairs formed per duplex) was determined by a comparison of melt profiles for the immobilized oligonucleotides to similar reported values and values for $dA_{20} + dT_{20}$ in solution.

The fraction of single strands present in the system at any temperature ($f_{ss}(T)$) may be determined through the use of the following equation:

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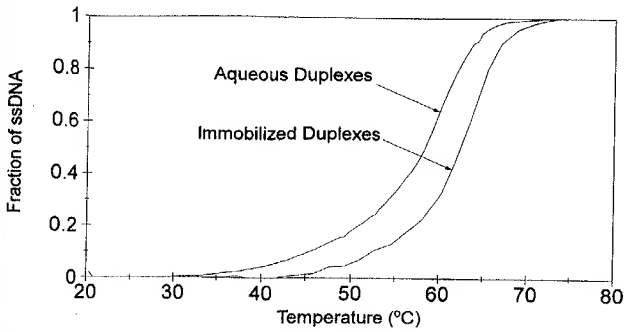

Figure 3. Thermal denaturation profiles of aqueous $dA_{20} + dT_{20}$ and immobilized dT_{20} with aqueous dA_{20}.

$$f_{ss}(T) = \frac{A(T) - A_{ds}(T)}{A_{ss}(T) - A_{ds}(T)} \quad (1)$$

where $A(T)$, $A_{ss}(T)$, and $A_{ds}(T)$ are the absorbances of the experimentally obtained melting curve, the upper baseline (single-stranded oligomers), and the lower baseline (double-stranded oligomers), respectively, at temperature T.[60] By plotting f_{ss} against temperature, the duplex melting temperature can be obtained by determining the temperature at which $f_{ss} = 0.5$.

Melt Curve Studies of Support-Bound Duplex DNA and Aqueous-Phase DNA. The purpose of the thermal denaturation studies was to examine whether linkage of an oligonucleotide to a solid support through a terminal nucleotide phosphate would cause the loss of degrees of freedom with respect to the availability of each nucleotide to partake in formation of the double-stranded structure. Melt profiles for the thermal denaturation of dsDNA immobilized on the surface of a quartz wafer and dsDNA in solution were obtained, and the results of these investigations are summarized in Figure 3. The duplex melting temperature of the immobilized strands with aqueous-phase complement strands was 62.4 ± 0.3 °C. The T_m value for the aqueous phase $dA_{20} + dT_{20}$ duplex was determined to be 60.5 °C using the software supplied by Varian. Kibler-Herzog et al.[61] have recently reported the melting temperature of a $dA_{19} + dT_{19}$ duplex in 1.02 M NaCl to be 61.1 °C. This suggests that for the immobilized oligomers investigated in this work, the extent of hybridization was complete with base pairing of 20 bases per strand. The small differences in the three T_m values may be accounted for by the facts that each of these experiments was done on a different instrument at different times and the salt concentration used in this work was slightly lower than that used by Kibler-Herzog et al. As the duplex stability in low ionic strength buffers is less than that in high ionic strength buffers, it would be expected that the melting temperature of the immobilized $dT_{20} + dA_{20}$ duplex would be higher in the buffer of lower stringency.[62] In addition to this, a greater value of T_m for the immobilized duplex than those of the aqueous-phase duplexes should not be considered unusual, as only one of the strands will experience a significant gain in entropy upon melting of the immobilized duplex. These factors lead to the conclusion that, within experimental uncertainty, the immobilized $dT_{20} + dA_{20}$ duplexes were more stable than, if not as stable as, the aqueous-phase $dA_{20} + dT_{20}$ and $dA_{19} + dT_{19}$ duplexes. This also suggets that no hindrance of duplex formation is observed with respect to the availability of the bases for hybridization. This result is in accord with the investigations of Wolf et al.,[43] in which oligonucleotides bound to solid supports via a long-chain aliphatic tether at the strand termini (3′-end) were not observed to be hindered with respect to hybridization efficiency. Future work will include monitoring hybridization with longer analyte strands (≥100 base pairs) to determine whether the nonhybridizing portions of the analyte strand will hinder duplex formation at the surface of the oligonucleotide-functionalized optical fibers.

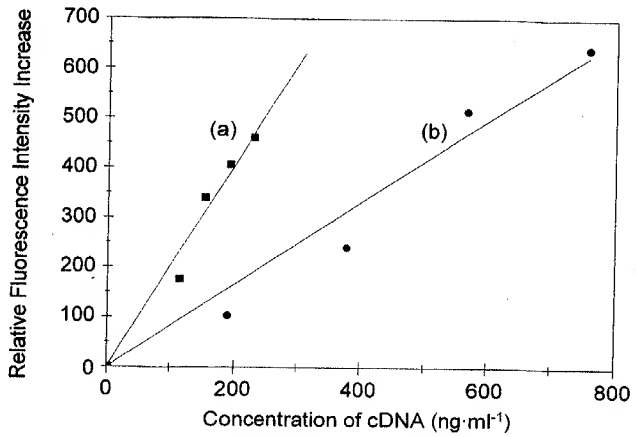

Figure 4. Sensitivity of optical sensors: (a) 11-month-old fiber which had been cleaned by sonication in ethanol and (b) 1-month-old fiber which was stored in vacuo and used without cleaning.

Response to Nucleic Acid Sequences. Fluorescence intensity values are reported as relative magnitudes, obviating the need to control experimental parameters such as laser intensity, optical alignment, and PMT gain. The robustness of the fibers, and of DNA as a biorecognition element, was made evident by the maintenance of activity after long-term storage and vigorous cleaning conditions. Fibers that were stored for over 1 year in vacuo, in 1:1 ethanol/water solutions, in absolute ethanol, or dry at −20 °C provided response characteristics identical to those of freshly prepared fibers. Adsorbed fluorescent contaminants which were accumulated through long-term storage were completely removed (as confirmed through fluorescence microscopy) by sonicating the fibers in a solution of 1:1 ethanol/water, with full maintenance of activity and sensitivity. Figure 4 shows the response of a 1-month-old fiber (stored in vacuo) used with no cleaning of the surface and an 11-month-old fiber (stored dry at −20 °C) which had been cleaned by sonication in ethanol solution. It should be noted that the sensitivity of the cleaned 11-month-old fiber is identical to that of 1-month-old fibers cleaned by the same procedure (data not shown). Cleaning of the sensor by sonication prior to its use has consistently been observed to increase the sensitivity of the device by a factor of ~2.5. A more detailed investigation of the response of the optical sensor to cDNA by staining with EB has been previously reported.[33] The sensors have provided femtomolar detection limits and a response which is linear with the concentration of cDNA (MW = 6199 g·mol^{-1}). The regression lines shown in Figure 4 show good fits to the data points, with r^2 values of 0.965 and 0.968 for the 1- and 11-month-old fibers, respectively. From these data, the sensitivity of the optical sensor (11-month-old) was determined to be an increase in fluorescence intensity of 203% per 100 ng·mL^{-1} of

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Table 1. Response of the Optical Sensor to Solutions Containing 570 ng·mL^{-1} cRNA, 570 ng·mL^{-1} cDNA, and 600 ng·mL^{-1} dR_{19}

sample	concn (ng·mL^{-1})	relative fluorescence intensity increase (%)
complement DNA (dA_{20})	570	615 ± 28
complement RNA (rA_{20})	570	683 ± 23
noncomplement DNA (dR_{19})	600	4 ± 23

cDNA, with a measured limit of detection of 86 ng·mL^{-1}. Maintenance of calibration has been observed for all experiments done thus far in which as many as five regenerations have been done over durations of up to 12 h.

The response of the sensor to complement RNA (cRNA) was also investigated. A 3.8 ng·μL^{-1} solution of rA_{20} (450 μL) was introduced into the cuvette containing hot hybridization buffer. This yielded a 570 ng·mL^{-1} solution of cRNA in the cuvette. A hybridization and staining procedure identical to that used for investigations of DNA–DNA hybridization on the surface of the sensor was followed. The results of this investigation are summarized in Table 1. As can be seen in Table 1, the responses of the optical sensor to DNA and to RNA agree to within the reported experimental error, while the fiber treated with a similar concentration of noncomplementary sequences provided essentially no response.

In our earlier work, incomplete removal of all the ethidium bromide and possibly some of the cDNA strands was observed to result in a small increase in the baseline intensity after each hybridization on the surface of the optical sensor.[33] As some of the fiber remained above the solution level in the cuvette, it was suspected that some of the EB and possibly some of the cDNA had diffused up and along the fiber surface. To determine if this was the case, the entire surface of the fiber was flushed with 20 mL of hot (85 °C) deionized water. The surface of the objective and the proximal terminus of the optical fiber were then carefully dried using a small piece of absorbent tissue (Kimwipe) to ensure that any changes of signal that were observed were not the result of changes in the optical coupling. This method of sensor regeneration was found to be effective, wherein the fluorescence intensity from the fiber after regeneration was observed to return to baseline values.

Response Time. Experiments were done to determine the response time of a single optical sensor (stored for 7 months in ethanol) to cDNA strands. All conditions for hybridization remained the same with the exception that the incubation times were varied. For each determination, injections of 30 μL of a 56.8 μg·mL^{-1} solution of aqueous dA_{20} were made. Cooling of the hot hybridization buffer containing the cDNA strands to 25 °C (over 10–40 min) was done by controlling the rate at which a cool (17 °C) solution of ethylene glycol was flushed through the jacket surrounding the cuvette. After each hybridization, complete regeneration of the fiber surface was observed. A summary of the results is shown in Figure 5, and indicates that 99% of full signal is achieved after an incubation time of 30 ± 3 minutes.

Effects of Ethidium Bromide Staining Time and Concentration. The effects of EB staining time and concentration were investigated in order to determine the parameters which would provide optimal sensor response. In the first set of experiments, the staining time of the sensor with EB was changed after each hybridization with cDNA. For each determination, injections of 30 μL of a 56.8 μg·mL^{-1} solution of aqueous dA_{20} were made, and the hot hybridization buffer in the cuvette which contained the cDNA strands was allowed to cool to room temperature over 30 min. A 1 mg·mL^{-1} solution of EB in water (10 μL) was added to the cuvette after each hybridization to provide an EB concentration of 8.4×10^{-3} M. The results of this experiment are summarized in Figure 5. A staining time of 20 min with 8.4×10^{-3} M EB was required to generate ≥99% of the full signal.

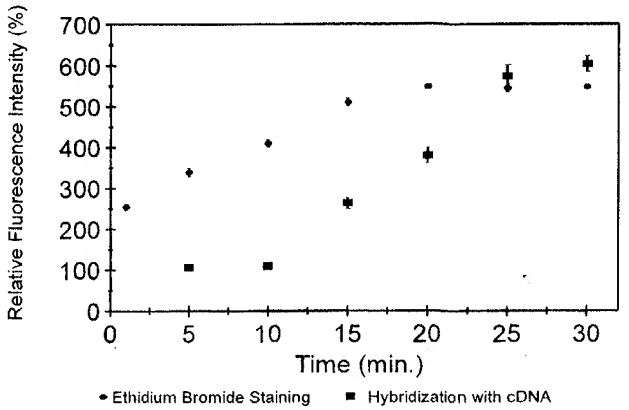

Figure 5. Hybridization and ethidium bromide staining times of the optical sensor.

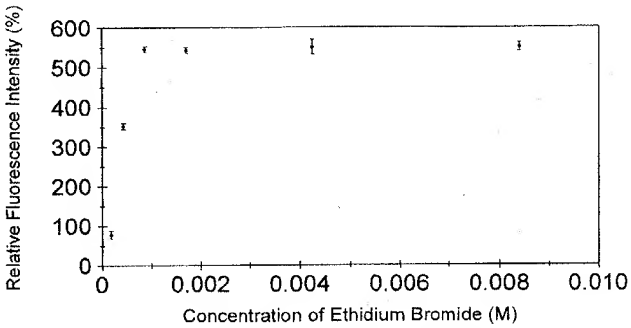

Figure 6. Response of the optical sensor to different concentrations of ethidium bromide during dsDNA staining.

Investigations were then done to determine the effect of EB concentration during the dsDNA staining time. All hybridization parameters were identical to those used in the study of EB staining time, and a staining time of 20 min was used for all determinations. As can be seen in Figure 6, staining with EB solutions of concentrations of 8.5×10^{-4} M or greater were required to generate ≥99% of the full signal, with a staining time of 20 min.

Stability of the Biosensor to Sterilization and Cleaning. The ability to clean and sterilize a bioprobe or biosensor device so that it may be usable in an on-line configuration is a significant advantage. As the specific binding properties of nucleic acids are based on secondary structure, the use of nucleic acids in biosensor fabrication leads to devices which are stable to not only prolonged storage but also to harsh washing conditions and sterilization. A summary of the effects of cleaning by sonication in absolute ethanol (15 min) and autoclaving (120 °C for 20 min at 4 atm pressure in sterile water) on the response of the sensors to (~400 ng·mL^{-1}) is shown in Table 2. Both sonication in ethanol and autoclaving are observed to improve the response of the sensor,

Table 2. Effect of Storage Conditions, Cleaning, and Sterilization on Sensor Response to 400 ng·mL^{-1} cDNA

storage conditions	cleaning/ sterilization conditions	relative fluorescence intensity increase (%)
1:1 ethanol/water (25 °C)		333 ± 20
95% ethanol (25 °C)		395 ± 20
dry (−20 °C)		341 ± 20
1:1 ethanol/water (25 °C)	autoclave	430 ± 20
1:1 ethanol/water (25 °C)	sonication	453 ± 20

most likely through the removal of contaminants on the surface of the sensor (stored dry for 11 months or stored in ethanol).

CONCLUSION

This work has demonstrated that ssDNA may be covalently immobilized onto the surface of quartz optical fibers and can undergo hybridization with complementary DNA and RNA introduced into the local environment of the sensor. Hybridization events may be detected by the use of the fluorescent DNA stain ethidium bromide, which is known to intercalate into dsDNA. The detection system was shown to be reproducible, regenerable, long-lived, and rugged. The response toward complementary sequences of DNA and RNA was identical within the reported experimental error. The response time of the sensor was found to be on the order of 50 min, wherein a hybridization time of 30 min and an EB staining time of 20 min was required in order to achieve 99% of the full analytical signal. Complete regeneration of single-stranded membranes was achieved by rinsing the entire surface of the sensor with hot (85 °C) deionized water. Melting studies of immobilized duplexes of $dT_{20} + dA_{20}$ have shown that the duplex melting temperature is 62.4 °C. By comparison with the melting studies of similar nucleic acid systems, it was determined that the extent of hybridization between the immobilized $dT_{20} + dA_{20}$ was complete, wherein all 20 bases on each strand were involved in hydrogen bonding through base pairing interactions.

The overall objective of this ongoing research effort is to create a working biosensor. In order to fulfill the definition of a biosensor, this system must be able to transduce the binding event without the requirement of an external solution treatment. In order to meet this requirement, we are immobilizing strands of DNA of mixed base sequence onto the surface of optical fibers where a tether of 6−40 atoms length is covalently attached to the 5′-end of each strand. Onto the end of the tether is attached a suitable fluorescent dye which is quenched in aqueous solution and shows intense luminescence when intercalated into the base stacking region of double-stranded DNA. This provides a reagentless sensor, yielding a true biosensor which can be internally calibrated by the presence of the covalently attached dye and can provide fast response. Our preliminary work has made use of EB tethered by a 19-atom chain and has been used to prepare sensors that have response times on the order of 5 min.

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